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Influence of Bilirubin on the Determination of Acid Phosphatase in Serum

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Summary: Bilirubin interferes greatly in the continuous measurement of acid phosphatase, using α-naphthyl phosphate as substrate and diazotized 2-amino-5-chlorotoluene (Fast Red TR) as the colorimetric reagent. Fast Red TR and the so-called "direct" (but not "indirect") bilirubin form a coloured azo-compound which absorbs light above 480 nm. Negative absorbance changes are observed at 405 nm, which is used in the determination of acid phosphatase. This leads to apparently negative activities.

Störung der Bestimmung der sauren Phosphatase im Serum durch Bilirubin

Zusammenfassung: Die kontinuierliche Messung der sauren Phosphatase mit α-Naphthylphosphat als Substrat und diazotiertem 2-Amino-5-chlortoluol (Fast Red TR) als Farbreagenz wird durch Bilirubin erheblich gestört. Sogenanntes "direktes" nicht jedoch "indirektes" Bilirubin bildet mit Fast Red TR ein farbiges Azoprodukt, das bei Wellenlängen jenseits von 480 nm absorbiert. Bei der Meßwellenlänge für die Reaktion der sauren Phosphatase (405 nm) kommt es zu einem Absorptionsabfall, so daß negative katalytische Aktivitäten vorgetäuscht werden.

Introduction

In 1971, *Hillmann* (1) described a continuous method for the determination of acid phosphatase in serum, which is now widely used in clinical laboratories (2,3). The procedure involves hydrolysis of α-naphthyl-phosphate by acid phosphatase and subsequent formation of a coloured azo-compound (4) from the resulting α-naphthol and diazotized 2-amino-5-chlorotoluene (Fast Red TR). It was pointed out by *Hillmann* (1) that bilirubin was not attacked by Fast Red TR under the conditions of the test. We, however, observed low or even "negative" values for acid phosphatase in icteric sera, suggesting a still unexplained interference of bilirubin or some bilirubin metabolite.

Materials and Methods

The following commercial reagents and test combinations were used:

bilirubin standard solution from DuPont, Wilmington, USA,

dimethylformamide from Merck, Darmstadt, Germany,

Fast Red TR from Serva, Heidelberg, Germany,

acid phosphatase test according to *Hillmann* (1) from Boehringer, Mannheim, Germany and from Smith and Kline Instruments, Palo Alto, USA,

bilirubin test according to *Wahlefeld* et al. (5) from Boehringer, Mannheim,

test for direct bilirubin according to *Jendrassik & Grof* (6) from Gödecke, Freiburg, Germany.

Measurements were performed on the photometer 1101 M from Eppendorf Gerätebau, Hamburg, Germany. Absorption spectra were recorded on the Hitachi 156 double-wavelength spectrophotometer.

Results

Figure 1 shows a typical acid phosphatase reaction curve (A) recorded at 405 nm wavelength and 37 °C with a non-icteric serum (bilirubin content 12 μmol/l, catalytic concentration of acid phosphatase 61 U/l). The sample volume is 0.05 ml and the total cuvette volume 0.6 ml in this experiment. When 10 to 50 μl of the reagent mixture are replaced by a highly icteric serum without measurable phosphatase

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activity (bilirubin content 856 µmol/l, direct bilirubin 616 µmol/l), the resulting activity (5th minute) is greatly decreased. Negative absorbance changes are observed during the first 4 minutes.

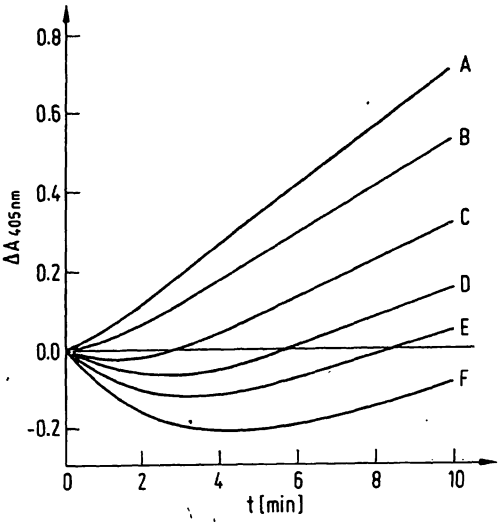

Fig. 1. Acid phosphatase determination (37 °C) in the presence of varying amounts of icteric serum. A = without icteric serum, B–F = 10, 20, 30, 40, and 50 µl icteric serum. For further details see text.

The decrease observed at 405 nm is accompanied by an increase at 546 nm (fig. 2) suggesting the formation of a red compound from yellow bilirubin in the presence of diazonium salt Fast Red TR. This is further established by the absorption spectra of an icteric serum before and after incubation with Fast Red TR (0.6 mg/ml in 0.2 mol/l citrate buffer, pH 5.2) in the absence of the phosphatase substrate α-naphthylphosphate. The absorption decreases between 380 and 479 nm and increases beyond these isosbestic points (fig. 3).

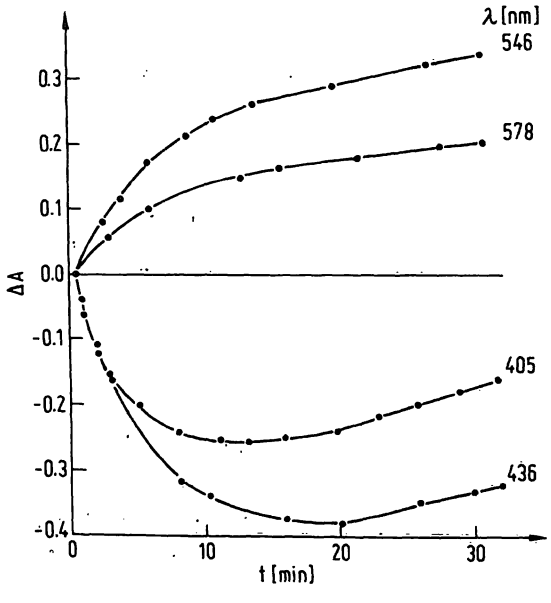

Fig. 2. Acid phosphatase assay, using an icteric serum (bilirubin 259 µmol/l, direct bilirubin 176 µmol/l). Measurements are performed at 405, 436, 546, and 578 nm at 37 °C. For further details see text.

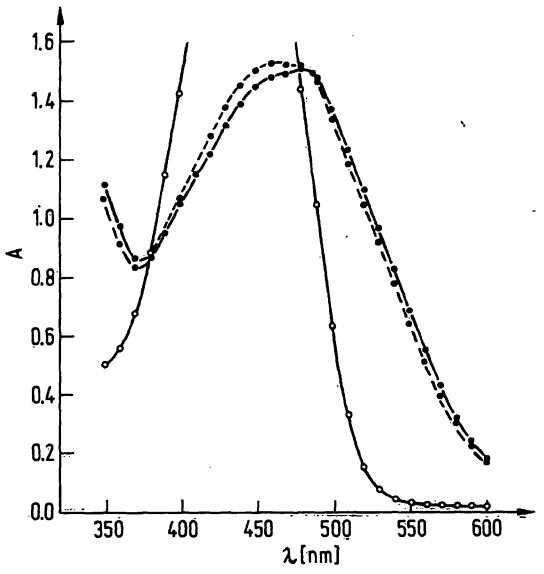

Fig. 3. Absorption spectra of an icteric serum diluted 21-fold in 0.2 mol/l citrate buffer, pH 5.2 before and after incubation with Fast Red TR (final concentration 0.6 g/l).
Open circles: before incubation
Filled circles: after incubation for 30 min (dotted line) and 60 min (solid line).

The interfering decrease of absorption is only observed with markedly icteric sera containing direct bilirubin (tab. 1). In contrast, normal and slightly icteric sera (direct bilirubin less than 50 µmol/l) produce a small increase of absorption at 405 nm during

Tab. 1. Reaction between direct bilirubin and Fast Red TR in the absence of α-naphthylphosphate. Each assay contained 0.5 ml citrate buffer (0.2 mol/l, pH 5.2), 0.3 mg Fast Red TR and 0.05 ml serum. Absorbances were read at 405 nm and "pseudoactivities" were calculated from the 5-min-value using a molar absorption coefficient of 14.1 × 10^3 l/mol × cm (4).

Total bilirubin (µmol/l)	Direct bilirubin (µmol/l)	ΔA_{405}/min recorded within the 1st min	5th min	10th min	Pseudo-activity (U/l)
27	9	0.008	0.002	0.000	6.2
31	14	0.006	0.003	0.000	2.3
39	17	0.007	0.002	0.000	1.6
58	36	0.008	0.005	0.000	3.9
91	50	0.002	0.000	0.000	0.0
139	84	−0.028	−0.008	0.000	−6.2
142	89	−0.050	−0.010	−0.002	−7.8
173	103	−0.045	−0.014	−0.005	−10.9

the first 3 to 5 minutes, even in the absence of a α-naphthylphosphate (tab. 1). This transitory "pseudoactivity" may be caused by an azo-coupling reaction between Fast Red TR and some serum components (4). At concentrations of direct bilirubin above 50 μmol/l the typical decrease of absorption is observed at 405 nm (tab. 1).

In contrast to water-soluble direct bilirubin no interfering reaction is observed with insoluble ("indirect") bilirubin, for example with a commercial bilirubin standard solution or with an icteric neonatal serum (fig. 4). The interfering reaction can, however, be started by addition of an organic solvent such as dimethylformamide (fig. 4).

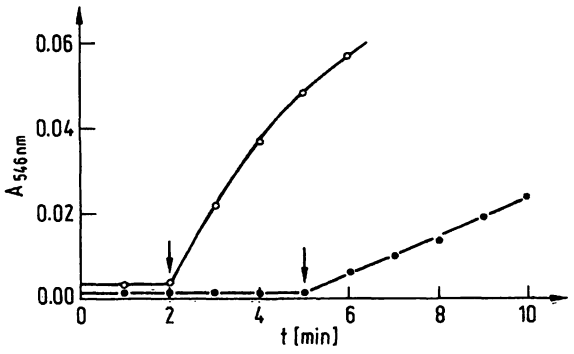

Fig. 4. Influence of dimethylformamide (final concentration 160 ml/l) on the reaction of "indirect" bilirubin with Fast Red TR.
Open circles: bilirubin standard solution (325 μmol/l)
Filled circles: neonatal serum (bilirubin 171 μmol/l, direct bilirubin 5 μmol/l).
The arrows indicate the addition of dimethylformamide to a mixture of 1 ml phosphatase reagent (without α-naphthylphosphate) and 0.1 ml sample.

Discussion

In 1977, *Shaw* et al. (2) reported an inhibiting effect of icteric sera on the acid phosphatase assay of *Hillmann* (1). The authors were, however, unable to demonstrate an influence of purified bilirubin, so that the "inhibiting" effect of icteric sera remained doubtful.

In this article, we show that bilirubin does not inhibit acid phosphatase but interferes with the indicator reactions by forming a coloured azo-compound. We have observed that this reaction is greatly dependent on assay conditions such as pH and presence of albumin or organic solvents (fig. 4). Under the conditions of the *Hillmann* assay only water-soluble bilirubin derivatives interfere with the phosphatase assay while insoluble bilirubin reacts only in the presence of organic solvents (fig. 4) or at pH-values around 7 (not shown).

At concentrations of direct bilirubin below 50 μmol/l this interference may be partly compensated by a "pseudoactivity" observed in non-icteric sera (4) while at higher concentrations direct bilirubin reduces the measurable phosphatase activity appreciably.

We have observed that the interfering effects of bilirubin are comparable in the presence and absence of tartrate which is added to the assay as an inhibitor of prostatic acid phosphatase (1). Therefore, by expressing prostatic phosphatase activity as the difference between values obtained with and without tartrate, the interference should, theoretically, be eliminated.

The following objections must, however, be considered:

1. Absorbance curves are non-linear in the presence of bilirubin (fig. 1) so that activity measurements become less precise.
2. Absorbance changes caused by bilirubin are usually higher than those induced by acid phosphatase. This enlarges the analytical error.

For these reasons we cannot recommend this kinetic method for the measurement of prostatic acid phosphatase in sera with direct bilirubin above 50 μmol/l.

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